Oxidation of Aminophenols by 4a-Hydroperoxy-5-ethyllumiflavin Anion. Flavoenzyme Hydroxylase Mechanism

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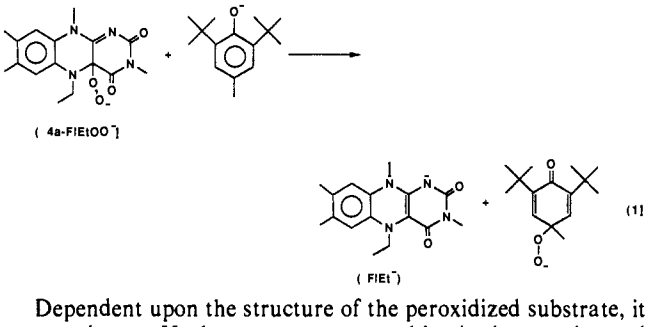
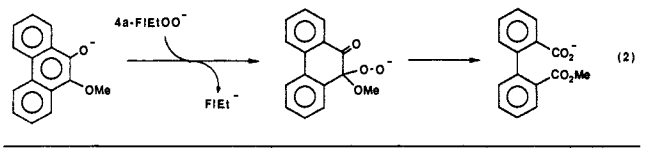
Abstract: Product and kinetic studies have been carried out (absolute *t*-BuOH solvent, O_2-free N_2 atmosphere, 30 °C) for the oxidation of a number of aminophenolate anions (*o*-, *m*-, and *p*-amino, 2-amino-5-methyl, 3-methyl-4-amino, *p*-*N*,*N*-dimethylamino) and aminonaphtholate anions (1-amino-2-naphtholate, 4-amino-1-naphtholate, 2-amino-3-naphtholate) by the hydroperoxy anion of 5-ethyl-3-methyl-4a,5-dihydro-4a-hydroperoxylumiflavin (4a-FlEt-OO^-). All but *m*-aminophenolate and 2-amino-3-naphtholate underwent oxidation in the stopped-flow time range. Under the pseudo-first-order conditions of [phenolate] or [naphtholate] $\gg$ [4a-FlEt-OO^-] the pseudo-first-order rate constant (k_{obsd}) exhibited saturation on increase of [phenolate] or [naphtholate]. The maximum value of k_{obsd} at saturation was independent of the structure of the oxidizable substrate, showing that 4a-FlEt-OO^- is endothermically converted to a species (X) that is trapped by phenolate or naphtholate anions. The rate constant for 4a-FlEt-$OO^- \rightarrow$ X ($\sim$0.3 s^{-1}) is, within experimental error, identical with that previously observed for the dioxygen-transfer reaction from 4a-FlEt-OO^- to a number of di-*tert*-butylphenolate and 3-methylindolate anions. *p*-Aminophenolate anion yields *p*-benzoquinone while *p*-(*N*,*N*-dimethylamino)phenolate provides both *p*-benzoquinone and (*N*,*N*-dimethylamino)-*p*-benzoquinone. Migration of the *N*,*N*-dimethylamino substituent in the formation of (*N*,*N*-dimethylamino)-*p*-benzoquinone is proposed to occur via a (*N*,*N*-dimethylamino)aziridine cation intermediate. Oxidation of *o*-aminophenolate and 5-methyl-2-aminophenolate provides the corresponding phenoxizanones. The stoichiometry of these oxidations are in accord with 4a-FlEt-OO^- + aminophenolate $\rightarrow$ $FlEt^-$ + *o*-quinone imine followed by condensation of *o*-quinone imine with remaining aminophenolate and a second oxidation by 4a-FlEt-OO^- to yield dihydrophenoxazinone. Dihydrophenoxazinone is then proposed to proceed to phenoxizanone by oxidation with HOO^-, which is a product of the initial oxidations with 4a-FlEt-OO^-. The mechanism of the flavoenzyme mixed function hydroxylation of phenolate anions is discussed in terms of the relative stereochemical disposition of enzyme bound flavin cofactor 4a-hydroperoxide and phenolate substrate—deduced from the X-ray coordinates of *p*-hydroxybenzoate hydroxylase with flavin and 3,4-dihydroxybenzoate bond at the active site.

The hydroperoxy anion of 4a-hydroperoxy flavins such as 5-ethyl-3-methyl-4a,5-dihydro-4a-hydroperoxylumiflavin (4a-FlEtOO$^-$) react with ambident phenolate and indole anions in the stopped-flow time range. The reactions result in the transfer of the peroxide dioxygen moiety to a carbon center of the substrate with the formation of a 1,5-dihydroflavin anion.[1-4] An example of this class of reactions is provided in eq 1.



(1)

Dependent upon the structure of the peroxidized substrate, it may undergo a Hock rearrangement resulting in ring opening and the formation of a dicarboxylic acid (eq 2) or the loss of HO^- to yield a quinone which on further reaction with 1,5-dihydroflavin anion coproduct is reduced to a hydroquinone (eq 3). Though there are no known flavoenzyme dioxygenases the reaction of eq 2 is a dioxygenation reaction. The reaction of eq 3 mimics the enzymatic phenol hydroxylation reaction. Oxidation by the transfer of an O_2 moiety from a hydroperoxide is unique in organic chemistry.



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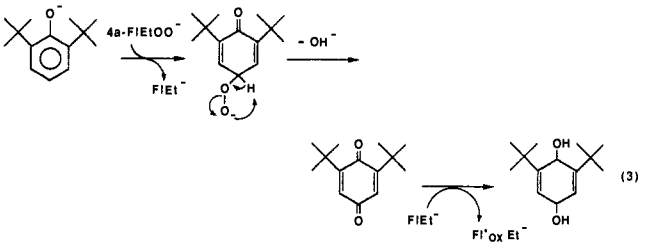

(3)

We present here the results of a study of the oxidation of aminophenolate ions by 4a-FlEtOO$^-$. The mechanism of hydroxylation of phenolate species by flavoenzyme mixed function hydroxylases is discussed in terms of the X-ray coordinates of the *p*-hydroxybenzoate hydroxylase enzyme–product structure.

Experimental Section

General. Spectrophotometric measurements were carried out on a Cary 118C spectrophotometer or a Durram stopped-flow spectrophotometer. ^1H NMR spectra were recorded on a Varian T60 with tetramethylsilane as internal reference and chemical shifts expressed as δ values. HPLC analyses were carried out with a Du Pont reverse-phase column (Lichosorb RP-8, 5 μm, 250 × 4.6 mm), using acetonitrile–water mixtures at a flow rate of 0.8–1.2 mL/min. IR spectra were recorded on a Perkin-Elmer 137 spectrophotometer using KBr pellets.

Materials. (All melting points are uncorrected.) *tert*-Butyl alcohol was distilled from CaH_2 under nitrogen. **Aminophenols** and **aminonaphthols** were obtained from Aldrich, recrystallized from acidic methanol or ethanol, and stored under nitrogen. ***p*-(*N*,*N*-Dimethylamino)-phenol** was prepared by the method of Stedman,[5] distilled under reduced pressure at 0.1 mmHg (bp 210 °C), allowed to crystallize, and kept in a dry box under N_2. The ^1H NMR in DMSO-d_6 showed a singlet at 3.67 ppm, two doublets at 6.92 and 7.77 ppm, and one broad peak at 10.17 ppm for the methyl, aromatic protons, and hydroxy proton, respectively. **N^5-Ethyl-4a-hydroperoxy-3-methyllumiflavin** (4a-FlEtOOH) has been synthesized in this laboratory in 85 to >90% purity:[1a,b] UV/vis λ_{max} = 370 nm (ϵ in *t*-BuOH = 8000 M^{-1} cm^{-1}). **2-Amino-3*H*-isophenoxazin-3-one** was prepared after a method of Nagasawa, Gutman, and Morgan.[6]

(1) Kemal, C.; Bruice, T. C. *J. Am. Chem. Soc.* **1979**, *101*, 1635.
(2) Muto, S.; Bruice, T. C. *J. Am. Chem. Soc.* **1980**, *102*, 4472.
(3) Muto, S.; Bruice, T. C. *J. Am. Chem. Soc.* **1980**, *102*, 7559.
(4) Muto, S.; Bruice, T. C. *J. Am. Chem. Soc.* **1982**, *104*, 2284.
(5) Stedman, E. *Biochem. J.* **1926**, *26*, 4031.

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Table I. Conditions and Wavelengths Monitored for the HPLC Analysis of Substrates and Products in the Reaction of the Hydroperoxy Anion of 4a-Hydroperoxy-N^5-ethyllumiflavin (4a-FlEtOO$^-$) with Various Substrates[a]

substrate[b]	products[c]	wavelength[d] (nm)	flow rate (mL/min)	retention time (min)
o-AP				6.50
	(2-Am)IPX	400	0.80	18.4
(5-Me)oAp		420	0.80	7.20
	(4-Am)IPX			16.5
p-AP[e]				2.90
	p-BQ	290	0.80	2.55
(3-Me)pAp		290	0.80	3.20
	(Me)pBQ			2.30
NNDMAp		290	0.80	16.4
	p-BQ	495	0.80	2.60
	(NNDMAm)pBQ			4.80
1,2-AN		257	1.00	3.50
	1,2-NQ			6.25
1,4-AN		325	0.80	3.00
	1,4-NQ			6.75

[a] Solvent employed MeCN/H_2O = 1:1, unless otherwise stipulated. [b] Abbreviations: *o*- and *p*-AP, *o*- and *p*-aminophenolate; (5-Me)oAp, 2-amino-5-methylphenolate; (3-Me)pAp, 3-methyl-4-aminophenolate; NNDMAp, *p*-(*N*,*N*-dimethylamino)phenolate; 1,2-AN, 1-amino-2-naphtholate; 1,4-AN, 4-amino-1-naphtholate. [c] Abbreviations: (2-Am)IPX, 2-amino-3*H*-isophenoxazin-3-one; (4-Am)IPX, 4-amino-1,7-dimethyl-3*H*-isophenoxazin-3-one; p-BQ, *p*-benzoquinone; (Me)pBQ, methyl-*p*-benzoquinone; (NNDMAm)pBQ, (*N*,*N*-dimethylamino)-*p*-benzoquinone; 1,2-NQ, 1,2-naphthoquinone; 1,4-NQ, 1,4-naphthoquinone. [d] Wavelengths are not necessarily the λ_{max} values for substrates or products. [e] Solvent MeOH/H_2O = 1:1.

The crude material was sublimed and recrystallized from aqueous MeOH: mp 253–255 °C (lit.[6] mp 254–256 °C); UV/vis (EtOH) λ_{max} 236 nm (2.9 × 10^4 M^{-1} cm^{-1}), 420 (2.45 × 10^4 M^{-1} cm^{-1}), and 435 (2.5 × 10^4 M^{-1} cm^{-1}) [lit.[8] λ_{max} 238 nm (2.92 × 10^4 M^{-1} cm^{-1}), 422 (2.44 × 10^4 M^{-1} cm^{-1}), and 437 (2.5 × 10^4 M^{-1} cm^{-1}). **4-Amino-1,7-dimethyl-3*H*-isophenoxazin-3-one** was prepared by a literature procedure.[6] Attempts at purification (sublimation, etc.) failed. The crude decomposition point was at ca. 260 °C; UV/vis (EtOH) λ_{max} 415 nm (ε 1.5 × 10^4 M^{-1} cm^{-1}); IR (CO stretching frequency) 1700 cm^{-1}. **2-Methylbenzoquinone** was synthesized according to Traylor and co-workers[7] by reaction of 2-methyl-1,4-hydroquinone with thallium(III) trifluoroacetate in trifluoroacetic acid: mp 66–67 °C (lit.[7] mp 67–68 °C); UV/vis λ_{max} (EtOH) 299 nm (ε 3.1 × 10^3 M^{-1} cm^{-1}). **2-(*N*,*N*-Dimethylamino)-1,4-benzoquinone** was synthesized by the method of Teuber and Hasselbach[8] and recrystallized from chloroform and diethyl ether: UV/vis λ_{max} 495 nm (ε 5754 M^{-1} cm^{-1}) and 322 (ε 347 M^{-1} cm^{-1}) [lit. λ_{max} 495 nm (5740 M^{-1} cm^{-1}) and 322 (347 M^{-1} cm^{-1})].

Product Analysis. Anhydrous and O_2-free *t*-BuOH was used as solvent. A 4a-FlEtOOH solution (10 mL, 6.10 × 10^{-5} M), 1 mL of substrate solution (1.01 × 10^{-3} M), and 0.5 mL of *t*-BuO$^-$K$^+$ (1.78 × 10^{-1} M) solution were mixed and stirred for 5 min, followed by acidification with 0.05 mL of glacial acetic acid. For the detection of the concentration of 1,5-dihydro-N^5-ethyllumiflavin (FlEtH) product, an aliquot of the acidified spent reaction solution was transferred to a Thunberg cuvette under a N_2 atmosphere and mixed with an excess of the nitroxyl radical 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl. The one-electron reduction of the nitroxyl radical by FlEt$^-$ provides the radical species FlEt' whose concentration was assayed at 640 nm (ε 5 × 10^3 M^{-1} cm^{-1}). The products formed from substrates were determined by HPLC analysis. Integrations of elution peaks were compared to plots of integration area vs concentrations by employing authentic samples of the products to determine the percentage yields of products (see Table I).

Reaction coordinate modeling for *p*-hydroxybenzoate hydroxylase is based upon the X-ray structure of the enzyme published by Schreuder, van der Laan, Hol, and Drenth[9] (Brookhaven Protein Database file 1PHH, 1988 edition) with oxidized flavin cofactor and the product 3,4-dihydroxybenzoate at the active site. Modeling and minimizations were done on a Silicon Graphics Iris 4D/70 computer graphics work station running QUANTA/CHARMm (Polygen Corp.). The Fl_{ox} fragment was that shown in the X-ray structure of *p*-hydroxybenzoate hydroxylase.[9]

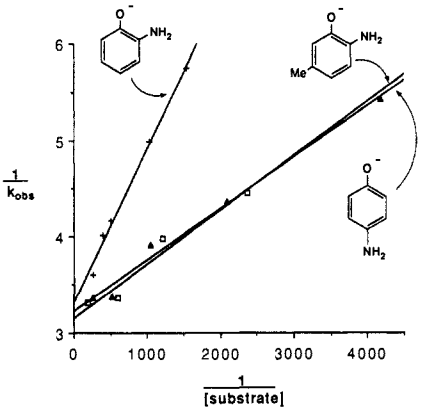


Figure 1. Representative plots of the reciprocal of the pseudo-first-order rate constants (k_{obsd}) for the reaction of phenolate anions with 4a-FlEt-OO$^-$ vs the reciprocal of the concentration of phenolate substrate.

Table II. Derived Rate Constants for the Reaction of Aminophenolate Species with the Anion of N^5-Ethyl-3-methyl-4a-hydroperoxyluminflavin (4a-FlEtOO$^-$)

substrate	k_1 (s^{-1})	$\alpha = k_2/k_3$ (M)
o-aminophenolate	0.33	4.8 × 10^{-4}
p-aminophenolate	0.32	0.17 × 10^{-4}
2-amino-5-methylphenolate	0.32	18.0 × 10^{-4}
4-amino-3-methylphenolate	0.32	2.2 × 10^{-4}
4-(*N*,*N*-dimethylamino)phenolate	0.20	0.52 × 10^{-5}

The structure of the flavin moiety substituted by an oxygen at the 4a-position was taken from the X-ray structure of 4a,5-(epoxyethano)-3-methyl-4a,5-dihydrolumiflavin.[10] Addition of a proton in accord with typical hydroxyl geometries provided the 4a-FlH-OH fragment, while addition of an oxygen atom to the oxygen at the 4a-position followed by adjustment of the O–O geometry, according to published X-ray structures of peroxides,[11] provided the 4a-FlH-OO$^-$ moiety. A hydrogen atom was then added to the latter in accord with typical hydroxyl geometries to provide the 4a-FlH-OOH fragment. *p*-Hydroxybenzoate and 1-hydroxycyclohexadien-6-one were taken as the best energy minimized structures by using the Abner minimization technique available within CHARMm. The stereoviews of the reaction sequence of Scheme III shown in Figure 2 were obtained by fitting the 4a-FlH-OH and 4a-FlH-OOH fragments as well as the *p*-hydroxybenzoate and 1-hydroxycyclohexadien-6-one species into the X-ray structure of *p*-hydroxybenzoate hydroxylase by least-squares fitting to similar atom types of the oxidized flavin cofactor and 3,4-dihydroxybenzoate that occupy the active site of the X-ray structure. In the least-squares fitting, only atom types that do not undergo changes in hybridization during the course of the reaction were superimposed. In the reaction of *p*-hydroxybenzoate with 4a-FlH-OOH, the position of the distal oxygen was adjusted to come as close to the benzoate ring as possible by continuously monitoring the distance of separation between the distal oxygen and the meta-carbon hydroxylation site while rotating the hydroperoxide O–O dihedral. The closest approach was when the C(10)–C(4a)–O–O dihedral was −160°, at a separation of 1.94 Å.

Results

The reactions of aminophenolate and aminonaphthoate anions with the hydroperoxide anion of 5-ethyl-3-methyl-4a-hydroperoxylumiflavin (4a-FlEtOO$^-$) have been studied by stopped-flow spectrophotometry by following the disappearance of 4a-FlEtOO$^-$ at 370 nm. On the stopped-flow bench, a *t*-BuOH solution of the 4a-FlEtOOH (~5 × 10^{-5} M) was mixed with a *t*-BuOH solution containing *t*-BuO$^-$K$^+$ and varying concentrations of the aminophenol. All *t*-BuOH solutions were kept dry and O_2 free (N_2 or Ar atmosphere), and reactions were carried out at 30 ± 0.2 °C. Aminophenol concentrations were greater than 10-fold that of

(6) Nagasawa, M. T.; Gutmann, H. R.; Morgan, M. A. *J. Biol. Chem.* **1959**, *234*, 1600.
(7) McKillop, A.; Swarm, B. P.; Traylor, E. C. *Tetrahedron* **1970**, *26*, 4031.
(8) Teuber, H. J.; Hasselbach, M. *Chem. Ber.* **1959**, *92*, 674.
(9) Schreuder, H. A.; van der Laan, J. M.; Hol, W. G. J.; Drenth, J. *J. Mol. Biol.* **1988**, *199*, 637.
(10) Bolognesi, M.; Ghisla, S.; Inoccia, L. *Acta Crystallogr.* **1978**, *B34*, 821.
(11) (a) Glidewell, C.; Liles, D. C.; Walton, D. J.; Sheldrick, G. M. *Acta Crystallogr.* **1979**, *B35*, 500. (b) Kass, D.; Oberhammer, H.; Brandes, D.; Blaschette, A. *J. Mol. Struct.* **1977**, *40*, 65. (c) Sax, M.; McMullan, R. K. *Acta Crystallogr.* **1967**, *22*, 281.
(12) Bruice, T. C. *Isr. J. Chem.* **1984**, *24*, 54.

Table III. Products of the Reaction of 4a-FlEtOO$^-$ with Amino-Substituted Phenolate and Naphthoate Species

substrate	percentage yield of products		
	FlEt$^\cdot$	FlEt$^-$	amine oxidation products
o-aminophenolate	76.9	10.7	80 (2-AmIPZ)
m-aminophenolate		no reaction	
p-aminophenolate	36.4	27.2	76 (*p*-BQ)
			<1 (unknown)
N,N-dimethyl-*p*-phenolate	22.3	30.1	85 (*p*-BQ)
			16 (DMA-*p*-BQ)
1-amino-2-naphtholate	80.6	9.0	89.1 (1,2-naphthoQ)
4-amino-1-naphtholate	68.7	17.8	76.0 (1,4-naphthoQ)
2-amino-3-naphtholate		no reaction	

the concentration of the flavin hydroperoxide and the concentration of *t*-BuO$^-$K$^+$ (2×10^{-2} M) was such that both flavin hydroperoxide and aminophenol or aminonaphthoate were maintained in the ionized state. All reactions were found to be pseudo-first-order up to at least 5 half-lives. Plots of the pseudo-first-order rate constants (k_{obsd}) vs the concentration of aminophenolate or aminonaphthoate substrate were found to be linear and of positive slope at low [substrate$^-$] and independent of [substrate$^-$] at the latter's higher concentrations. Reciprocal plots according to eq 4 were found to be linear. From the intercepts and slopes of plots (Figure 1) of $1/k_{obsd}$ vs $1/$[substrate$^-$] the values of k_1 and α (Table II) were calculated.

$$1/k_{obsd} = 1/k_1 + \alpha/k_1[\text{substrate}^-] \quad (4)$$

Product analysis was carried out on reactions with initial concentrations of [4a-FlEtOOH] = 2.7×10^{-4} M, [substrate] = $5\text{-}10 \times 10^{-3}$ M, and [*t*-BuO$^-$K$^+$] = 2×10^{-2} M. As in the kinetic runs, *t*-BuOH solutions were dry and O_2 free (N_2 or Ar atmosphere), and the temperature was maintained at 30 ± 0.2 °C. Reaction products and their yields are included in Table III.

The stoichiometry of the reaction of *o*-aminophenolate ion with 4a-FlEtOO$^-$ to provide 2-amino-3*H*-isophenoxazin-3-one is as shown in eq 5. The 77% yield of FlEt$^\cdot$ and 80% yield of 2-amino-3*H*-isophenoxazin-3-one are anticipated if about 80% of the 4a-FlEtOO$^-$ species is consumed in the formation of 2-amino-3*H*-isophenoxazin-3-one. 2-Amino-3*H*-isophenoxazin-3-

$$2\ o\text{-}H_2N\text{-}C_6H_4O^- + 2\ \text{4a-FlEtOO}^- \rightarrow \text{2-Am-IPZ} + \text{FlEt}^- + \text{Fl}_{ox}\text{Et}^+ \quad (5)$$

$$\text{FlEt}^- + \text{Fl}_{ox}\text{Et}^+ \rightarrow 2\ \text{FlEt}^\cdot$$

one was characterized by comparison of its spectrum to that of an authentic sample (λ_{max} 422 and 437 nm, Experimental Section). Oxidation of 3-methyl-4-aminophenolate by 4a-FlEtOO$^-$ provides 4-amino-1,7-dimethyl-3*H*-isophenoxazin-3-one. The identification of the product as an isophenoxazinone is based on the characteristic position of the IR C=O stretching frequency (1700 cm^{-1}) and visible spectrum (λ_{max} in EtOH at 415 nm, ϵ 14800 M^{-1} cm^{-1}). The isophenoxazinones have very characteristic UV/vis spectra around 400–500 nm and generally have IR absorbance at ~1600 cm^{-1}.[7]

Discussion

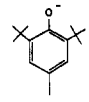
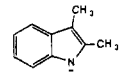
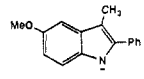
The dynamics and products of dioxygen transfer, from the hydroperoxy anion of 5-ethyl-3-methyl-4a,5-dihydro-4a-hydroperoxylumiflavin (4a-FlEtOO$^-$) to a number of ambident phenolate and pyrrole anions, have previously been investigated under the pseudo-first-order condition of [substrate$^-$] ≫ [4a-FlEtOO$^-$]. Regardless of the structure of the reactive substrate, the reactions shared in common a first-order dependence on [substrate$^-$] at low substrate concentrations and zero-order dependence upon [substrate$^-$] at higher substrate concentrations (i.e., saturation in substrate$^-$). This behavior is characteristic of reactions which occur by either (i) equilibrium formation of a complex or intermediate on bimolecular reaction of reactants followed by formation of products, or (ii) equilibrium conversion of the reactant at limiting concentrations to a reactive intermediate that then reacts with the second reactant that is present in excess and whose concentration is varied. For either case a plot of the reciprocal of the pseudo-first-order rate constant ($1/k_{obsd}$) vs $1/$[substrate] (eq 4) should be linear. For (i), it can be shown that the intercept would be equal to the reciprocal of the rate constant for break down of the complex or covalent intermediate formed from substrate$^-$ and 4a-FlEtOO$^-$ to products. This rate constant should be dependent upon the structure of the substrate anion. In the case of (ii), the intercept would represent the reciprocal of the rate constant for the endothermic conversion of 4a-FlEtOO$^-$ to an intermediate which is then trapped by substrate$^-$. Thus, for (ii) the intercept is independent of the nature of the substrate. Plots of the reciprocal of the pseudo-first-order rate constant ($1/k_{obsd}$) vs $1/$[substrate$^-$] (eq 4) with substrate$^-$ representing a number of ambident phenolate and pyrrole anions are linear, and the intercept is independent of the structure of the substrate$^-$. These results support the reaction sequence of eq 6. The experimental eq 4 pertains

$$\text{4a-FlEtO}_2^- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{X} \xrightarrow{k_3[\text{substrate}^-]} \text{FlEt}^- + \text{substrate-O}_2^- \quad (6)$$

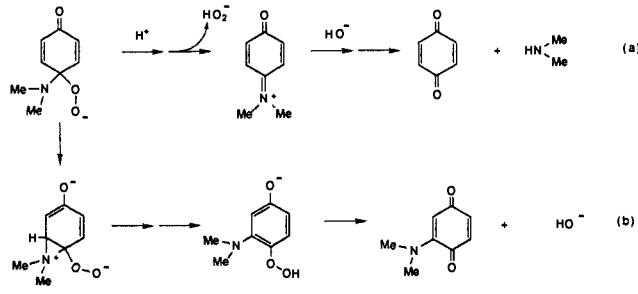
to the reactions of eq 6 where $\alpha = k_2/k_3$. Pertinent kinetic results from past studies are provided in Table IV. Examination of Table IV shows that $k_1 = 0.36 \pm 0.02$ s^{-1} while the partition coefficient k_2/k_3 varies from 2.2×10^{-4} to 130×10^{-4} M. In accord with eq 6, k_1 is independent of the nature of substrate$^-$ while k_2/k_3 is strongly dependent. Examination of the results of the present study (Table II) shows that the kinetics for the reaction of aminophenolate and aminonaphthoate species with 4a-FlEtOO$^-$ also support the involvement of the reaction sequence of eq 6. For *o*-aminophenolate, *p*-aminophenolate, 2-amino-5-methylphenolate, and 4-amino-3-methylphenolate, the average value of $k_1 = 0.32$ s^{-1} while k_2/k_3 varies from 0.17×10^{-4} to 18.3×10^{-4} M.

The formation of benzoquinone from *p*-aminophenolate and both benzoquinone and (*N,N*-dimethylamino)benzoquinone from *p*-(*N,N*-dimethylamino)phenolate (Table III) may find explanation in the sequences of Scheme I. Scheme I can also pertain to the formation of methylbenzoquinone on oxidation of 4-amino-3-methylphenolate (Table I).

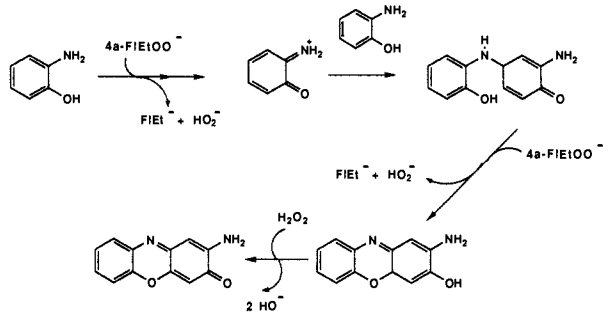
Table IV. Derivable Rate Constants for the Reaction of Phenolate and Indole Anions with 4a-FlEtOO$^-$[12]

substrate	k_1 (s^{-1})	k_2/k_3 (M)
	0.36	2.2×10^{-4}
	0.37	2.8×10^{-4}
	0.39	6.0×10^{-4}
	0.33	83.0×10^{-4}
	0.37	130×10^{-4}

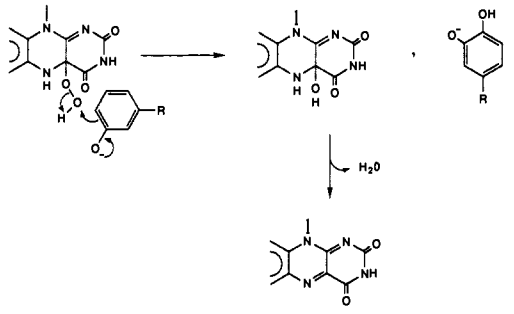
Scheme I



Scheme II

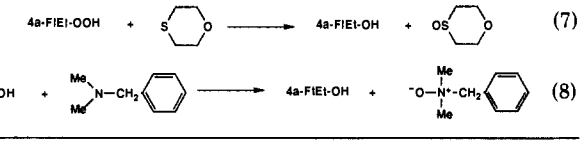


Scheme III



The oxidation of *o*-aminophenolate and 5-methyl-2-aminophenolate provides phenoxizanones. The stoichiometry of the reactions (Results Section) establishes that 2 equiv of 4a-FlEtOO$^-$ are consumed in the oxidation of *o*-aminophenol to provide 2-amino-3*H*-isophenoxazin-3-one. A likely mechanism[13] (Scheme II) involves the consumption of 1 equiv of 4a-FlEtOO$^-$ in the oxidation of *o*-aminophenolate to *o*-quinone imine and Michael addition of *o*-aminophenolate (present in excess of 4a-FlEtOO$^-$) to *o*-quinone imine, followed by a second 2e$^-$ oxidation with 4a-FlEtOO$^-$ and an intramolecular Michael addition to provide dihydrophenoxazinone. The 2 equiv of 4a-FlEtOO$^-$ consumed in the reaction provide 2 equiv of both HOO$^-$ and N^5-ethyl-1,5-dihydroflavin (FlEt$^-$). One equivalent of HOO$^-$ may be employed in the oxidation of the dihydrophenoxazinone species to the phenoxozanone product (dihydrophenoxazinone is instantly oxidized in air[14]) and the second equivalent of HOO$^-$ is used in the 2e$^-$ oxidation of FlEt$^-$ → FlEt$_{ox}^+$ (see eq 5). Much the same mechanism may be written for the oxidation of 5-methyl-2-aminophenolate by 4a-FlEtOO$^-$.

The Mechanism of Hydroxylation of Phenolate Substrates by Flavin Mixed Function Oxidase Enzymes To Provide Catechols.[15-17] It has been shown, in both model[18] and enzymatic[19] systems, that nucleophilic attack upon the distal oxygen of undissociated flavin hydroperoxides (as 4a-FlEt-OOH) leads to facile monooxygen transfer (eqs 7 and 8). Thus, a reasonable mechanism for the



(7)

(8)

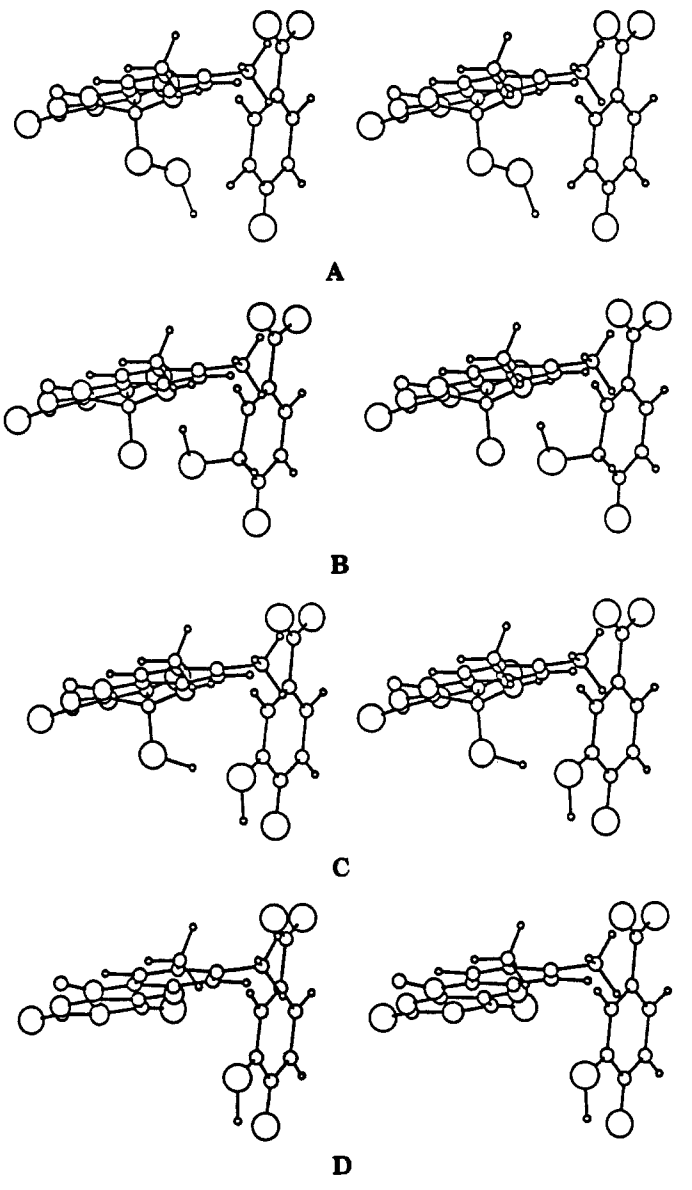
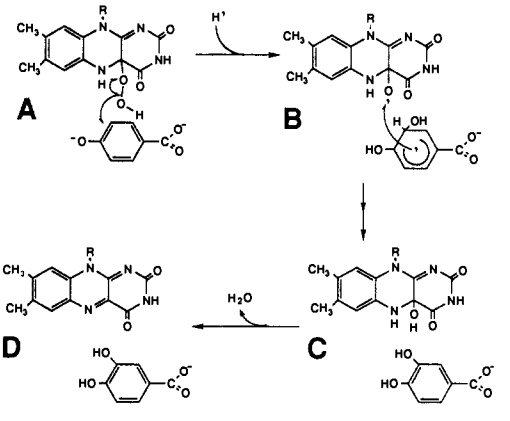


Figure 2. Three-dimensional structures of the reactants, intermediates, and products for the hydroxylation of *p*-hydroxybenzoate dianion (according to the steps of Scheme III) at the active site of *p*-hydroxybenzoate hydroxylase. Structure D represents the X-ray coordinates for the products located at the active site of *p*-hydroxybenzoate hydroxylase. Structures A, B, and C have been computer built from structure D as described in the Experimental Section.

Scheme IV



enzymatic hydroxylation of phenolate anions would involve nucleophilic displacement by phenolate anion on the distal oxygen of the enzyme bound 4a-FlH-OOH moiety (Scheme III).

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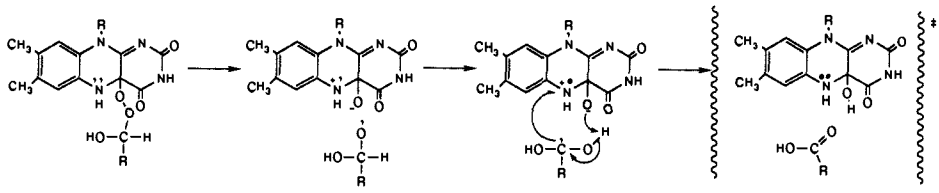
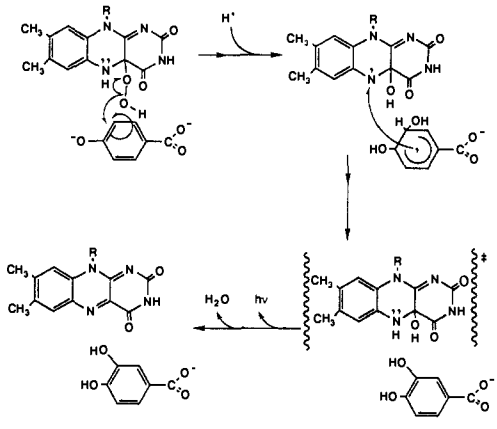
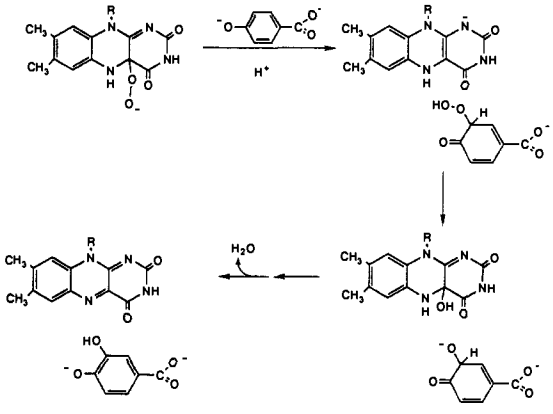
Scheme V



Scheme VI



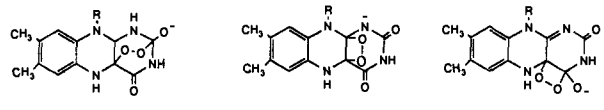
Scheme VII



Computer model building of the hypothetical intermediates for the hydroxylation of *p*-hydroxybenzoic acid by *p*-hydroxybenzoate hydroxylase has been carried out by employing the X-ray coordinates for the enzyme (see the Experimental Section). The three-dimensional structures are displayed in Figure 2. Examination of Figure 2 shows that the stereochemical disposition of *p*-hydroxybenzoate substrate and flavin 4a-hydroperoxide at the active site is completely in accord with the reaction sequence of Scheme III.

In the hydroxylation of certain alternate substrates by *p*-hydroxybenzoate hydroxylase an intermediate with λ_{max} of ~390 nm is observed. The spectrum of this intermediate was assigned[20] to a 6-amino-5-oxo-3*H*,5*H*-uracil (product of ring opening of the 4a-FlH-OH species between N(5) and C(4a)). The involvement of such an intermediate and the associated mechanism was disproved.[21] A radical mechanism has also been proposed (Scheme IV). Scheme IV is based upon the observation[22] that HO• adducts of *p*-hydroxybenzoate exhibit λ_{max} values between 360 and 410 nm. Examination of Figure 2 shows that such a mechanism is allowed; indeed it is the $1e^-$ version of the mechanism of Scheme III. A favored mechanism for the chemiluminescence (CL) observed with certain bacterial flavin monooxygenases (quantum yield 0.2) is shown in Scheme V.[23] With the assumptions that the flavin *p*-hydroxybenzoate hydroxylase reaction does provide a intermediate HO• adduct of phenolate species and that the mechanism of Scheme V is correct; the *p*-hydroxybenzoate hydroxylase reaction might be expected to exhibit CL since it possesses the required steps[24] of a CIEEL mechanism (Scheme VI). This feature has probably never been looked for.

The question remains as to whether the mechanism for the flavin phenol hydroxylase enzymes involves dioxygen transfer from a 4a-hydroperoxy flavin anion (4a-FlH-OO^-) as shown in Scheme VII. The details of the mechanism of dioxygen transfer to phenolate anions by 4a-FlEtOO^-, in model systems, is not known since the structure of the common intermediate X (eq 6) is unknown. The possibility that the endothermically formed intermediate X represents the solvent separated species (FlEt^- + $^3O_2$), (Fl$Et^•$ + $O_2^{•-}$) or (FlEt^- + $^1O_2$) has been examined and it has been concluded that these could not represent X.[1-4] If this is so, then it is reasonable to conclude that the dioxygen and flavin components of X must be intimately associated as a solvent-caged pair or compound. The covalent adducts [which can not be the isomeric 10a-hydroperoxyflavin anion[25] (i.e., 10a-FlEt-OO^-)] **1**, **2**, and **3** have been considered.[12,26] Examination of Figure 2



1 **2** **3**

establishes that neither **1**, **2**, nor **3** could be involved in the *p*-hydroxybenzoate hydroxylase reaction. If the intermediate X (eq 6) in the model reactions could be represented by either of the solvent-caged pairs (Fl$^•$, $O_2^{•-}$) or (FlEt^-, $^3O_2$) the mechanism of Scheme VII would be plausible (eqs 9 and 10, where S^- = phenolate).

$$(FlH^- O_2 S^-) \rightarrow (FlH^- O_2^{•-} S^•) \xrightarrow{H^+} (FlH^- HOOS) \rightarrow (4a\text{-}FlH\text{-}OH\ ^-OS) \quad (9)$$

$$(FlH^• O_2^{•-} S^-) \rightarrow (FlH^- O_2^{•-} S^•) \xrightarrow{H^+} (FlH^- HOOS) \rightarrow (4a\text{-}FlH\text{-}OH\ ^-OS) \quad (10)$$

Acknowledgment. This work was supported by the National Institutes of Health.

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